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Article

Molecular and Associative Evaluation of *ERG11*, *ERG3*, *CDR1*, and *SNQ2* Genes Linked to Fluconazole Resistance in *Candida glabrata* in a Colombian Population

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Abstract: The resistance of *Candida* spp to fluconazole is a serious problem; Therefore, it must be investigated to expand the understanding of the phenomenon with a view to its clinical applicability. The aim was to evaluate the expression of *ERG3*, *ERG11*, *CDR1*, and *SNQ2* genes involving computational analysis. Relative expression was quantified by RT-qPCR. Metrics were obtained by molecular docking and through Fisher's discriminant functions, a predictive classification against susceptibility to fluconazole was performed. The expression of *ERG3*, *CDR1*, and *SNQ2* genes was higher in resistant strains than in dose-dependent sensitive strains. The gene with the highest expression in strains exposed to fluconazole was *CDR1* and, in both resistant and dose-dependent sensitive strains exposed to fluconazole, the *ERG11* gene had lower expression. The molecular docking model generated lower median fluconazole-*ERG11* contacts than the median fluconazole-*ERG3*, -*CDR1*, and -*SNQ2* contacts. The predicted classification through the multivariate model for fluconazole sensitivity obtained an accuracy of 73.5%. Resistant strains had a significant expression of *CDR1*, *SNQ2* and *ERG3* genes, compared to a comparatively low expression of *ERG11*. Molecular analysis identified a low affinity between fluconazole and its drug target, which may explain a lower intrinsic susceptibility of the fungus.

Keywords: *Candida glabrata*; fungal drug resistance; reverse transcriptase polymerase chain reaction; fluconazole; molecular docking simulation; multivariate analysis

1. Introduction

Candida spp is an etiological agent frequently isolated as a cause of infections [1,2]; a situation that represents a health problem that must be successfully managed at the population care level; additionally, the approach to infections generated by *Candida* spp represents a challenge associated with pharmacological resistance to drugs that are usually considered first line in the antifungal therapeutic arsenal and that correspond to the so-called azoles, a group represented by fluconazole [3,4].

Resistance can be caused by multiple factors, for example, frequent exposure of the fungus to antifungal agents, where its ability to evolve and adapt to hostile environments ensures its survival. In addition, the exposure of the biological agent to the conventional mechanisms of action of the drugs and the lack of new antifungal active principles with alternative mechanisms of action, make the treatment complex given the growing resistance, which is due to adaptive biological structural and functional adjustments of the fungi, which translates into a decrease in pharmacological effectiveness [3,5].

Typically, the mechanism of action of the azoles is the blockage of the cytochrome P450 enzyme complex associated with lanosterol 14- α -desmethylase. This enzyme converts lanosterol into the fungal wall structural intermediate ergosterol. From a molecular perspective, the free nitrogen atom located in the azole ring binds to the iron atom of the heme group of the enzyme lanosterol 14- α -desmethylase, and it is precisely in the enzymatic pocket of the complex where *Candida* induces structural changes that limit the access of azoles to the protein [6].

A mechanism associated with resistance involves the extrusion of drugs from inside the fungal cell; in which proteins or pumps belonging to two superfamilies, ATP Binding Cassette, and MFS, which are encoded by genes *CDR1*, *SNQ2*, *PDH1*, and *MDR* respectively, intervene [7–16]. Another mechanism of resistance is associated with alterations in the expression and mutations of genes encoding enzymes involved in the ergosterol biosynthesis pathway, such as lanosterol 14- α -desmethylase and C-5 desaturase, which is responsible for synthesizing toxic sterols that accumulate in the fungus when exposed to azoles. The *ERG11* gene encodes for the enzyme lanosterol 14- α -desmethylase and its overexpression and/or mutations are associated with resistance whose biological basis responds to the maintenance of the integrity of the fungal membrane in the presence of azole and to the decrease in the affinity between the azole and the enzyme that serves as a pharmacological target. On the other hand, mutations in the *ERG3* gene coding for the C-5 desaturase enzyme have been described as mechanisms associated with resistance, because the enzyme loses activity and stops synthesizing toxic sterols in the presence of azoles, which facilitates the survival of the fungus; At the same time, activation of synthesis routes of constituent compounds of the fungal membrane has been found [17–20].

Overall, the mechanisms described allow the fungus to avoid or reduce the effectiveness of azoles, a situation that implies the need for research to improve the understanding of the phenomenon from a molecular perspective and to make the problem visible to advance in its comprehensive approach. In this study, using reverse transcriptase polymerase chain reaction (RT-qPCR), the expression of *ERG3*, *ERG11*, *SNQ2*, and *CgCDR1* genes of *C. glabrata* was evaluated and high-level computational and data analysis routes were used to contribute to the understanding and explanation of the phenomenon of interest.

2. Materials and Methods

2.1. Type of Study

A general experimental approach was carried out, with a contribution to the explanatory level.

2.2. Strains and sample handling conditions

Sixteen strains of *C. glabrata*, collected in a previous study conducted in an intensive care unit in the city of Manizales by the Biosalud Research Group of the Universidad de Caldas, were used; these strains were tested to confirm the species and determine their viability.

2.3. Sample selection and analysis groups

Gene expression experiments followed the guidelines proposed in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. The strains were plated on potato dextrose agar (PDA, Scharlau Microbiology) and identification was performed on the Vitek 2 compact (Biomérieux). Then, each strain was tested for antifungal susceptibility using the protocols proposed by The Clinical and Laboratory Standards Institute (CLSI) [21]. According to the results obtained, the strains were classified into two susceptibility groups: Dose-Dependent Sensitive with a MIC of $< 32 \mu\text{g/mL}$ and resistant with a MIC of $64 \mu\text{g/mL}$.

2.4. Macrodilution assay with fluconazole and without fluconazole

Each strain was subjected to a macrodilution assay according to the protocol proposed by CLSI, liquid Sabouraud was used as the culture medium. All tubes were incubated at 35°C until the

logarithmic phase of yeast growth occurred, at which time each tube was read to establish its minimum inhibitory concentration, the size of the cell pellet for each fluconazole concentration was compared with the positive control, and the cells were collected from the tube in which cell growth was greater than 50%; The cell concentration was adjusted to a value between 2×10^8 and 3×10^8 yeasts/mL, a process that was also carried out with the tube corresponding to the positive control. The tubes were centrifuged to obtain the yeast sediment, which was stored in Eppendorf tubes free of DNase and RNase, adding RNA later (RNAaseZap solution, Ambion - RNA technology), to be kept at $-80\text{ }^{\circ}\text{C}$ until the moment of RNA extraction.

2.5. RNA extraction

To obtain RNA, the protocol proposed in the extraction kit used (RiboPure - Yeast Kit, Ambion RNA technology) was followed and the manufacturer's recommendations were followed.

2.6. RNA extraction quality criteria

The solution with RNA was subjected to 1% agarose gel electrophoresis to observe the integrity of the genetic material, the RNA was considered not degraded when two bands appeared in the gel. Measurement of RNA purity was performed on the UVIS Drop UVS99™ spectrophotometer (Avans Biotechnology) with absorbance readings of samples at 230, 260, and 280 nm. Those RNAs with a 260:280 and 260: 230 ratios greater than or equal to 2 were considered to be of high purity and were subsequently used in RT-qPCR.

To assess the presence of DNA after DNAase I treatment, PCR amplification of one of the RNA samples was performed without reverse transcription, and in the presence of a universal primer for *C. glabrata*, it was evaluated whether there was residual DNA amplification. DNA-contaminated strains were subjected to a new DNAase I treatment.

2.7. Standardization of the protocol

For standardization, the *C. albicans* ATCC 90028. strain was used as a reference for a sensitive strain and a strain from the fluconazole-resistant group was randomly selected. All procedures were performed according to previously established guidelines to ensure adequate purity of the reactions and thus avoid contamination with RNA from other microorganisms.

2.8. Reverse Transcription (RT) Protocol

The reverse transcription process was performed on the StepOnePlus Real-Time PCR System thermal cycler (Applied Biosystems), with the following protocol: incubation at 25°C for 10 minutes, then at 42°C for 15 minutes, and finally, the enzyme was inactivated at 85°C for 5 minutes. The RNA obtained was converted to cDNA using the SensiFast™ cDNA kit (Bioline). The manufacturer's instructions were carefully followed, with no modifications to the protocol.

2.9. Selection and optimization of primers for qPCR

Table 1 describes the primers for the reaction for both the reference endogenous control gene and the resistance genes (*ERG3*, *ERG11*, *SNQ2*, and *CgCDR1*) [22–24].

Table 1. Primer sequence.

GENE		Primer sequence (5'–3')
<i>CgCDR1</i>	Forward	CATACAAGAAACACCAAAGTCGGT-3'
	Reverse	GAGACACGCTAACGTTACACCAC-3'
<i>ERG11</i>	Forward	TCGGTCCATCTCTGTTTCTT
	Reverse	GAACACTGGGGTGGTCAAGT
<i>ERG 3</i>	Forward	AAGCGTGTGAACAAGGAC
	Reverse	GCGTAGGTCTTCTCTGTGA

SNQ2	Forward	CGTCCTATGTCTTCCTTACACCATT
	Reverse	TTTGAACCGCTTTTGTCTCTGA
URA3	Forward	GAAAACCAATCTTTGTGCTTCTCT
	Reverse	CATGAGTCTTAAGCAAGCAAATGT

2.10. qPCR experiment

Each of the assays of samples with and without exposure to fluconazole was performed with triple identical repeats on the StepOnePlus Real-Time instrument using the PowerUp SYBR Green Master Mix kit (Applied Biosystem). The specificity of the products of the q-PCR reactions was evaluated by analysis of the dissociation curves (Melting Curve).

Each q-PCR quantification reaction contained: 5 µL PorweUp SYBR Green Master Mix [2X], 0.8 µL of each primer pair, at a final concentration of 0,8 µM, 1 µL of DNAc (DNAc concentration adjusted to 1 ng/µL), the volume was made up to 10 µL with RNase- and DNase-free Water. The program consisted of an initial denaturation in two steps: 50°C for two minutes and 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and an extension at 60°C for 1 minute. In order to establish the DNAc dissociation temperature and identify nonspecific amplification reactions, the following parameters were used: 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds.

2.11. qPCR reaction analysis

Analysis of the reactions was performed by establishing the difference between the detection threshold crossing cycle (Ct), which exists between the resistance genes analyzed (and the reference endogenous control gene, for which *C. albicans* ATCC 90028 was used as the reference strain. Gene expression was quantified according to RQ (Relative Quantification). The analysis of the results was performed in the StepOne Plus Real-Time PCR software (Applied Biosystems). This same software allowed us to evaluate the efficiency of each of the reactions.

2.12. Molecular coupling

The molecular docking procedure was carried out using amino acid sequence and tertiary structure prediction for *ERG3*, *ERG11*, *CDR1*, and *SNQ2* (Uniprot codes P50860, P50859, Q6FK23 and Q6FQN3). The maximum resolution of the available crystal structures corresponded to 2.0 Å. Protein models were downloaded from the online database *CANDIDA*-GENOME DATABASE with structural prediction coupled to the ALPHAFOLD program [25,26]. The formats .mol2 for fluconazole and .pdb for the protein structures of interest were used. The analysis generated by the AVOGADRO program version 1.2.0 [27] was used for energetic optimization of the substrate. The exploratory docking process was developed with the open-use programs PyRx-AutoDock Vina version 0.8 and CHIMERA version 1.16 [28]. Molecular outcome metrics were presented with the Delta-Gibbs metric or Gibbs free energy (ΔG kcal/mol).

2.13. Statistical analysis

According to the normality tests of the results of the relative expression of each gene of interest, parametric (T-Student) or nonparametric (Wilcoxon Ranges) statisticians were used to determine differences in means or medians. An alpha (α) of 0.05 was established for the level of statistical significance.

The multivariate analysis was based on Fisher's linear discriminant function pathway for the presentation of a predictive classification score for sensitivity (resistant or sensitive dose-dependent) of *C. glabrata* [29,30]. The variables entering the multivariate model corresponded to gene, strain, mean RQ without fluconazole, and mean RQ with fluconazole. This route proposed the generation of qualitative variables with a numerical value associated with the category (dose-dependent sensitive = 1, resistant = 2; *ERG3* gene = 1, *CDR1* gene = 2, *SNQ2* gene = 3; strain 04-1A = 1, strain 07-1A = 2, strain 109-1A = 3, etc.). Numerical variables (Mean RQ with and without fluconazole) were

assumed at their continuous values in the model. Statistical significance was considered with predictive model classification accuracies (discriminant function) $\geq 70\%$. Biostatistical analysis was supported with the SPSS-licensed licensed-IBM package version 26 [31].

3. Results

3.1. Results of the identical triple qPCR experiment with and without exposure to fluconazole

Table 2 and Figure 1 below show the results of the identical triplicate assays of the qPCR experiment.

Table 2. Results of the identical triplicate assays of the qPCR experiment.

GENE	Experiment	RQ No exposed		RQ Exposed	
		Median	Medium	Median	Medium
CDR1	First	0,59	1,09	0,31	1,39
	Second	0,59	1,09	0,31	1,39
	Third	0,59	1,09	0,31	1,39
ERG11	First	0,23	0,7	0,07	0,31
	Second	0,23	0,7	0,07	0,31
	Third	0,23	0,7	0,07	0,31
ERG3	First	3,03	8,58	1,89	11,56
	Second	3,03	8,58	1,89	11,56
	Third	3,03	8,58	1,89	11,56
SNQ2	First	0,21	1,15	0,05	2,15
	Second	0,21	1,15	0,05	2,15
	Third	0,21	1,15	0,05	2,15

1. RQ data without normal distribution. 2. RQ data with normal distribution.

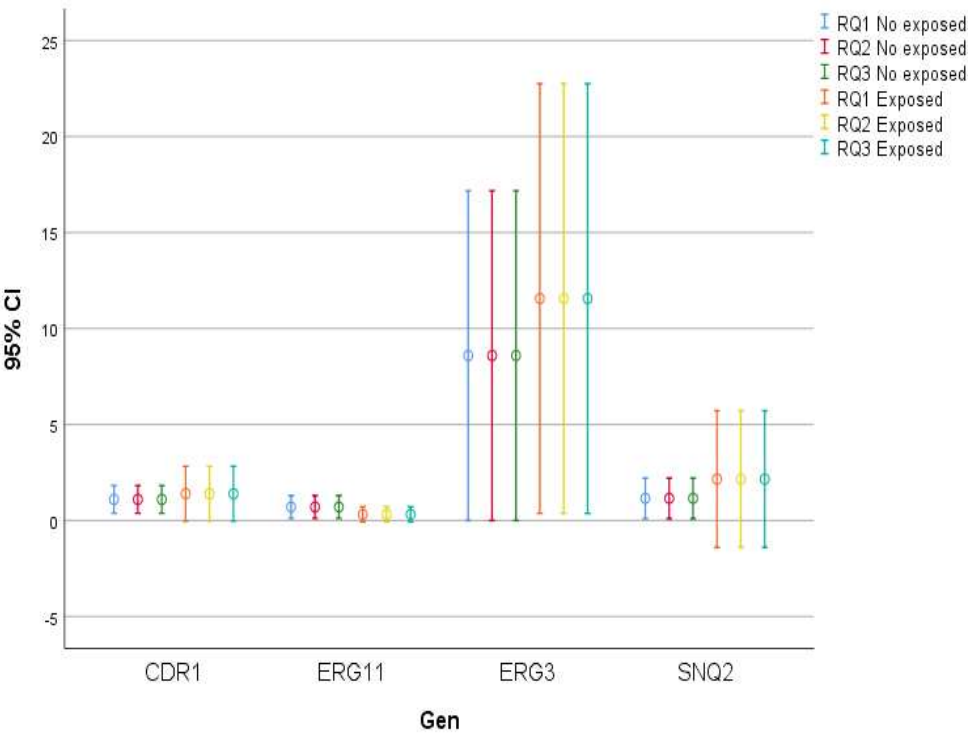


Figure 1. Error bars. Triple identical qPCR assays with and without exposure to fluconazole. The figure of error tables is introduced, especially useful for the representation of data that follow a

normal distribution, such as the RQ of the *ERG11* gene. This is because the traditional figure for representing data that do not follow a normal distribution, such as box-and-whisker plots, does not allow accurate visualisation due to the wide dispersion of the RQ values in the *CDR1*, *ERG3* and *SNQ2* genes.

3.2. Relative expression analysis

As a result of fluconazole sensitivity testing, the 16 strains were classified as follows: 6 were found to be resistant (Samples: 06-3A, 95-1A, 116-5, 137-1, 184-1A, 65-3) and 10 dose-dependent sensitive (Samples: 11-3A, 04-1A, 16-5A, 24-5A, 25-1A, 20-2A, 14-5A, 07-1A, 68-4, 109-1A). For the analysis of the results of the relative expression of the *CDR1*, *ERG11*, *ERG3*, and *SNQ2* genes, the relative expression value of the endogenous *URA3* gene, was used as a reference. Additionally, by parametric and non-parametric analysis routes, the medians and/or averages of relative gene expression were compared based on the condition of exposure or not to fluconazole, to determine differences in the level of expression.

As shown in Figure 2, the relative expression (RQ) of the genes studied in the 16 strains, under the condition of exposed and non-exposed to fluconazole, independent of the antifungal sensitivity profile, indicated that the relative expression of the exposed strains was higher than that of the non-exposed strains, in *ERG3* (RQ difference between exposed - unexposed strains=1.61 p=0.776), followed by *SNQ2* (RQ difference exposed - unexposed strains= 1 p=0.044) and finally *CDR1* (RQ difference exposed - unexposed strains= 0.3 p=0.523). On the contrary, the *ERG11* gene expression was higher in strains exposed to fluconazole than in non-exposed strains (RQ difference between exposed and non-exposed strains = -0.38, p=0.034).

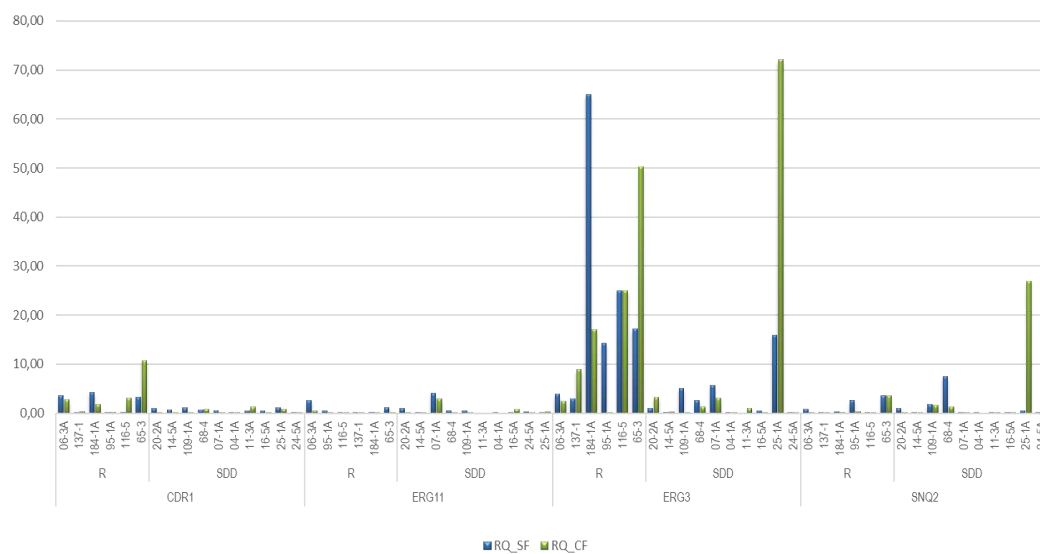


Figure 2. Relative expression of *CDR1*, *ERG11*, *ERG3* and *SNQ2* genes. Strains exposed Vs Strains not exposed to fluconazole.

Out of the 6 strains classified as resistant to fluconazole, independent of antifungal exposure, all overexpressed at least one gene and, interestingly, the *ERG3* gene was overexpressed in all of them, the *CDR1* gene was overexpressed in 4 (06-3A, 184-1A, 116-5 and 65-3), the *ERG11* gene in 2 (06-3A and 65-3) and the *SNQ2* gene in 2 strains (95-1A, 65-3).

The strain 65-3 had a higher relative expression of *CDR1* and *ERG3* genes than other strains, in contrast with a very low relative expression of *ERG11* and, additionally, under the condition of no exposure to fluconazole, it overexpressed the 4 genes object of this study, while the same strain exposed to the antifungal did not overexpress the *ERG11* gene. A similar phenomenon occurred with

strain 06-3A, which without exposure overexpressed the *CDR1*, *ERG11*, and *ERG3* genes and upon exposure to the antifungal did not overexpress the *ERG11* gene.

The descriptive analysis and the results of the parametric and nonparametric tests on the relative expression of the genes under study are reported in Table 3.

Table 3. Relative expression values of *CDR1*, *ERG11*, *ERG3*, and *SNQ2* genes according to sensitivity profile and antifungal exposure.

Analyzed Gen	Antimycotic Exposure	Resistant					Sensitive Dose-dependent				
		One-tailed analysis					One-tailed analysis				
		Media	Median	Minimum	Maximum	p	Media	Median	Minimum	Maximum	p
<i>CDR1</i>	Fluconazole-free	1,91	1,76	0,05	4,22	0,231 ¹	0,6	0,59	0,04	1,09	0,046 ¹
	With Fluconazole	3,15	2,22	0,24	10,75		0,33	0,11	0,01	1,33	
<i>ERG11</i>	Fluconazole-free	0,75	0,35	0,01	2,6	0,065 ²	0,67	0,22	0	4,06	0,086 ²
	With Fluconazole	0,12	0,04	0,01	0,49		0,43	0,08	0	2,97	
<i>ERG3</i>	Fluconazole-free	21,39	15,77	2,93	65	0,343 ¹	3,09	0,71	0	15,9	0,399 ¹
	With Fluconazole	17,28	12,94	0,01	50,3		8,1	0,66	0,01	72,12	
<i>SNQ2</i>	Fluconazole-free	1,22	0,52	0,01	3,59	0,014 ¹	1,1	0,12	0	7,4	0,166 ¹
	With Fluconazole	0,69	0,11	0	3,59		3,03	0,05	0	26,95	

1. Wilcoxon signed-rank test: One-tailed. 2. Student's t-test: One-tailed.

As shown in Table 3 and Figure 3, the relative expression of the *CDR1* gene in the resistant strains exposed to fluconazole was higher than in those not exposed; however, this difference was not statistically significant (Exposed: 2.22 - Not exposed: 1.76. p= 0.231). Dose-dependent sensitive strains exposed to fluconazole were found to have lower relative expression than non-exposed strains; this difference was statistically significant (RQ-Dose-dependent sensitive: Exposed: 0.11 - Non-exposed: 0.59. P=0,046).

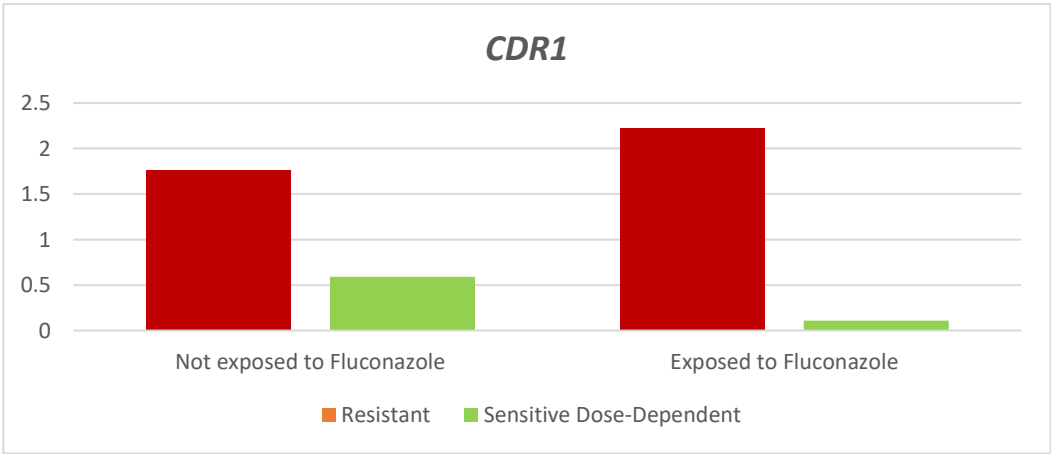


Figure 3. Relative expression of the *CDR1* gene according to the sensitivity profile and exposure to fluconazole.

According to the classification according to the sensitivity profile, a higher median QR was obtained in the resistant strains, both in those exposed and not exposed to fluconazole, than in the dose-dependent sensitive strains.

The relative expression of the *ERG11* gene in the dose-dependent resistant and sensitive strains was lower in those exposed to fluconazole than in those not exposed; however, this difference was not significant (RQ-Resistant: Exposed: 0.12 - Unexposed: 0.75. $p = 0.065$. Dose-dependent RQ-Sensitive: Exposed: 0.43 - Not exposed: 0.67. $p = 0.086$). Between the two groups according to the sensitivity profile, there was a higher average QR in the dose-dependent sensitive strains than in the resistant strains (Table 3 and Figure 4).

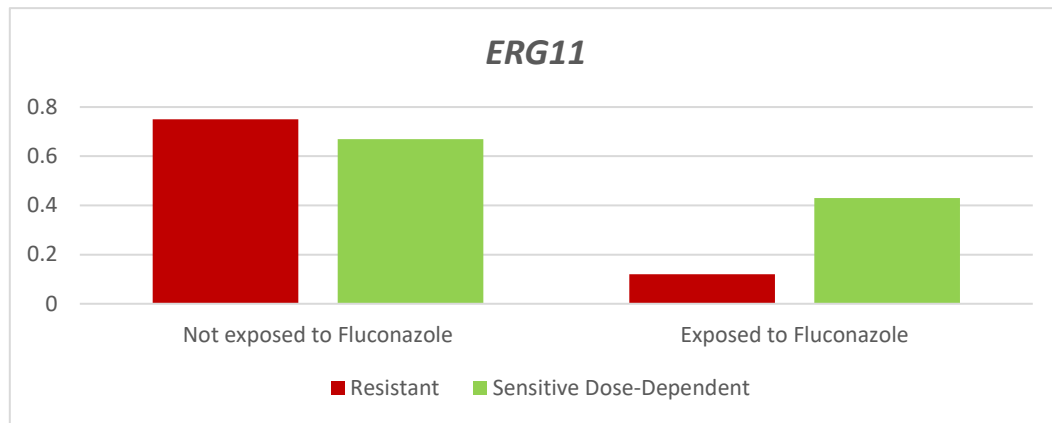


Figure 4. Relative expression of the *ERG11* gene according to sensitivity profile and exposure to fluconazole.

On the other hand, the relative expression of the *ERG3* gene in the dose-dependent resistant and sensitive strains exposed to fluconazole was lower than in the non-exposed strains, with statistically non-significant differences (RQ-Resistant: Exposed: 12.9 - Unexposed: 15.77 $p = 0.343$. RQ- Dose-dependent sensitive: Exposed: 0.66 - Not exposed: 0.71. $p = 0.399$). There was a higher median QR in the resistant strains, both exposed and not exposed to fluconazole than in the dose-dependent sensitive strains, as shown in Table 3 and Figure 5.

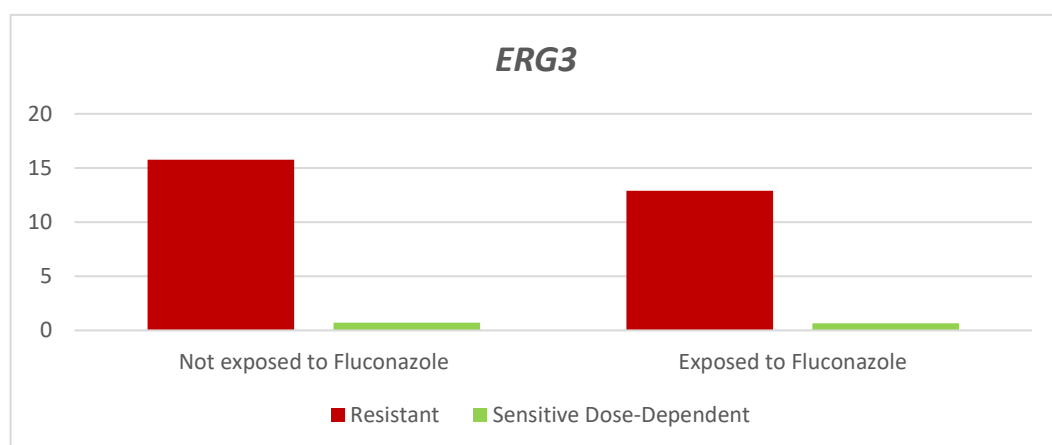


Figure 5. Relative expression of the *ERG3* gene according to the sensitivity profile and exposure to fluconazole.

Finally, the relative expression of the *SNQ2* gene in the resistant and dose-dependent sensitive strains was lower in the strains exposed to the antifungal agent; however, the difference was only significant for the resistant group (QR- Resistant: Exposed: 0.11 - Not exposed: 0.52 $p = 0.014$. QR- Dose-dependent sensitive: Exposed: 0.05 - Not exposed: 0.12. $p = 0.166$). The median QR was higher

in both exposed and unexposed resistant strains compared to dose-dependent sensitive strains (Table 3 and Figure 6).

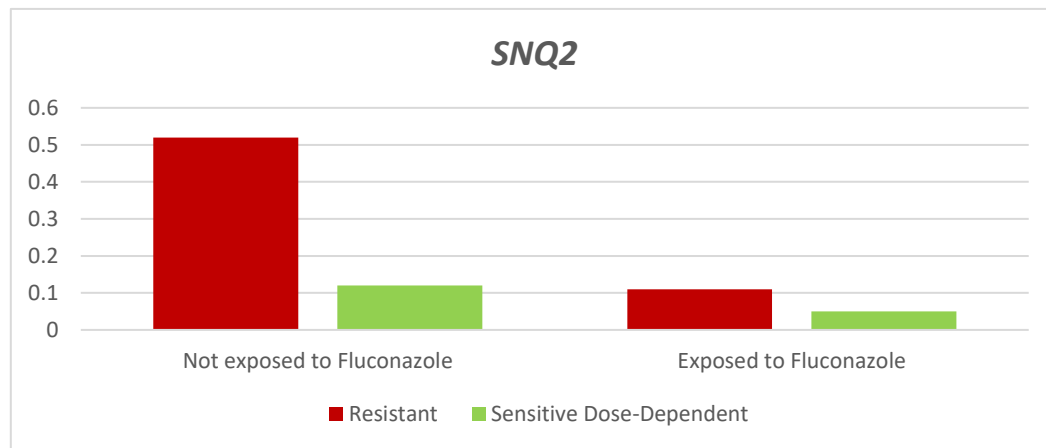


Figure 6. Relative expression of the *SNQ2* gene according to the sensitivity profile and exposure to fluconazole.

3.3. Results of the predictive model on the susceptibility profile

Multivariate evaluation by Fisher's discriminant function route generated the following coefficients for susceptibility profile classification (Table 4).

Table 4. Coefficients in the classification function for the dose-dependent sensitive (DDS) or resistant profile.

GENERAL SENSITIVITY PROFILE		
Model variables	Resistant	DDS
Analyzed gene	4,141	3,730
Categorized strain	0,369	0,276
Mean-RQ Fluconazole-Free	0,363	0,302
Media RQ with fluconazole	E	0,042
Constant	-8,009	-5,820

The overall comparative classification result between the original finding of the sensitivity profile by macrodilution testing versus the classification predicted by the model generated classification accuracy in 73.5% of cases.

The use of the coefficients is described in Table 5 below. If the findings in the research correspond to the *CDR1* gene (categorical value = 2), from *C. glabrata* strain 07-1A (categorical value = 2), with a mean RQ without fluconazole of 0.57 and a mean RQ with fluconazole of 0.22; they are replaced with the product of the coefficients and the products are then added together.

Table 5. Use of coefficients in the ranking function for dose-dependent sensitive (DDS) or resistant (DDS) profile.

Model variables	Sensitivity profile		Forecasting profile	
	Resistant	DDS	Resistant	DDS
Analyzed Gen	4,141	3,730	$2 \times 4,141$	$2 \times 3,730$
Categorized strain	0,369	0,276	$2 \times 0,369$	$2 \times 0,276$
Mean-RQ Fluconazole-Free	0,363	0,302	$0,57 \times 0,363$	$0,57 \times 0,302$
Media RQ with fluconazole	0,111	0,042	$0,22 \times 0,111$	$0,22 \times 0,042$
Constant	-8,009	-5,820	$1 \times -8,009$	$1 \times -5,820$

Total	1,2425	2,3738
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The total values are compared, and the upper value defines the predicted profile; in this case, the DDS category is predicted under the characteristics of the example. This classification is considered correct (accuracy) by the model in 73.5% of the cases.

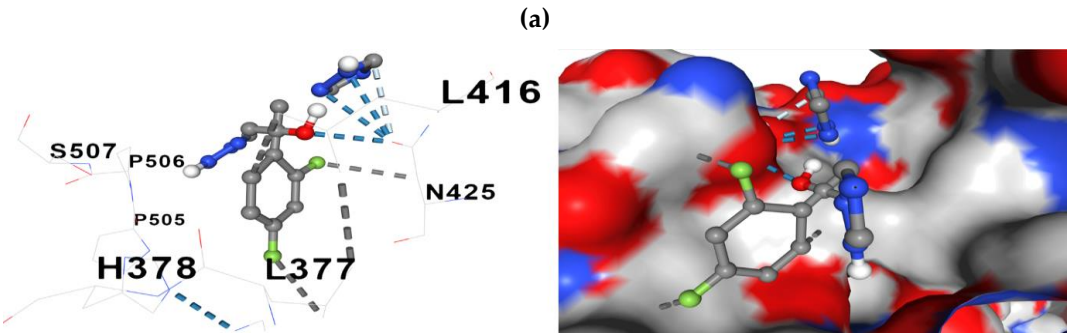
3.4. Molecular Coupling

Molecular processing using basic structural bioinformatics techniques (rigid docking) allowed us to obtain the ΔG values for the relationship between the proteins expressed from *C. glabrata* *ERG3*, *ERG11*, *CDR1*, *SNQ2*, and the substrate fluconazole. Table 6 describes the findings of the energetic molecular metrics for the main positions (interaction site) between fluconazole and the mentioned proteins.

Table 6. Representation of the ΔG (kcal/mol) for the various positions generated by the docking model between fluconazole and the proteins of interest in *C. glabrata*.

Fluconazole			
Protein	Model	ΔG (Kcal/mol)	Number of contacts
ERG3	1.	-6,2	19
	2.	-6,1	13
	3.	-6,0	6
	4.	-5,9	11
	5.	-5,8	6
ERG11	1.	-2,8	12
	2.	-2,6	11
	3.	-2,5	11
	4.	-2,4	10
	5.	-2,2	9
CDR1	1.	-7,0	14
	2.	-6,4	14
	3.	-6,4	15
	4.	-6,2	14
	5.	-6,2	11
SNQ2	1.	-7,6	17
	2.	-7,5	12
	3.	-7,4	12
	4.	-7,2	11
	5.	-7,1	11

The docking model further revealed the relationships between the constituent amino acids and fluconazole at the docking site. Two representative models of the process are presented in Figure 7: fluconazole - *ERG11* and fluconazole - *CDR1*.



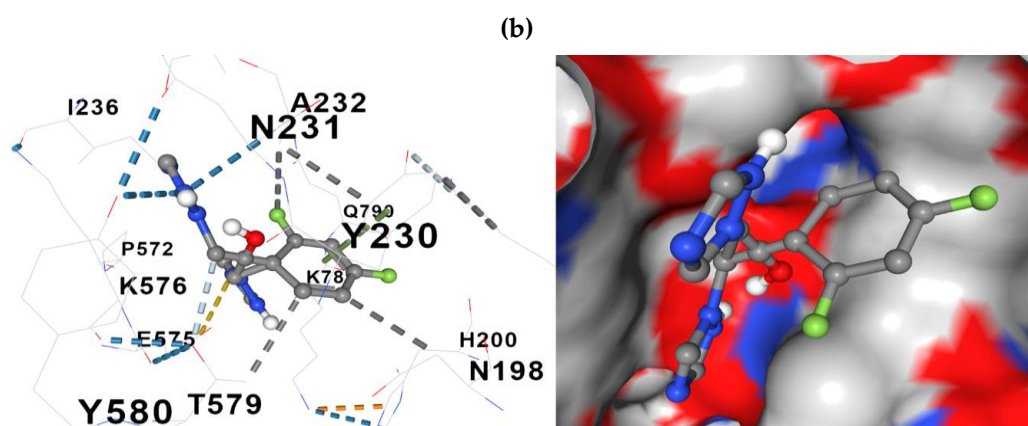


Figure 7. Comparative representations of the fluconazole - protein of interest docking. (a) 3D presentation of the molecular relationship (docking) between fluconazole and *ERG11*; (b) Presentation in 3D format of the molecular relationship between fluconazole and *CDR1*. Hydrophobic interactions with residues are shown with dashed gray lines and with gray surfaces on the protein.

A median number of detected contacts for the fluconazole-*ERG11* interaction of 10 and the fluconazole-*CDR1*, -*ERG3*, -*SNQ2* interaction, a median of 13, stand out in the docking results.

4. Discussion

This study addressed the quantification of the expression of some genes present in the species *C. glabrata*, which encode either for pharmacological efflux transporters or for enzymes involved in the ergosterol biosynthesis pathway.

The genes coding for efflux transporters addressed in this study were *CDR1* and *SNQ2*, and their expression findings allowed us to identify that regardless of the sensitivity profile to fluconazole, the strains exposed to the antifungal had higher relative expression than those not exposed, so that there was a greater amount of pharmacological efflux transporters willing to decrease the intracellular concentrations of fluconazole in the experimental group intervened with the antifungal; In addition, it was possible to identify that the resistant strains also had a higher expression than those classified as dose-dependent sensitive.

In a complementary perspective, the findings from the molecular perspective allow us to identify that the detected phenotype of activity of *C. glabrata*, establishes a limited pharmacological response in the scenario of overexpression of the *CDR1* gene and that the affinity between fluconazole-*SNQ2* is superior concerning the rest of the genes of interest in this study; and although no other research was found in which computational techniques were used to analyze molecular coupling, other studies with similar results were found, in which it has been determined that azole resistance is related to the overexpression of genes that encode for efflux pumps, such as the *CDR1* and *SNQ2* genes that encode for proteins of the ATP- Binding Cassette (ABC) superfamily [7,10,18,32–37].

Researchers Karl-H, Joseph-N and Tomas-E conducted a study on resistance in *C. albicans* and identified that deletion of the *ERG11* gene was associated with a positive regulation of *ERG3* expression, as well as the presence of enzyme inhibitor drugs and genetic lesions in ergosterol biosynthesis caused an increase in *ERG3* mRNA levels [38], although this finding does not correspond to the same species, it may biologically behave in a similar way in the *Candida* genus, since in our study we were able to identify in general very low *ERG11* expression values, most of them even lower than 1, which corresponds to the expression of the reference strain, in contrast to much higher *ERG3* expression values; also, our results showed overexpression of *ERG3* in almost all strains, both resistant and dose-dependent sensitive, a situation that could be extrapolated to lower susceptibility to the action of fluconazole; however, *ERG3* expression was highlighted by very high values in those classified as resistant compared to dose-dependent sensitive strains; it is plausible that *ERG3* overexpression is an early cellular stress response to try to maintain membrane integrity in the

presence of azole-generated blockade of the enzyme encoded by *ERG11*, particularly in *C. glabrata*, which is intrinsically less susceptible to fluconazole, a finding consistent with molecular docking results that indicate limited affinity between fluconazole and *ERG11* [39–41].

The available studies have focused on investigating loss-of-function mutations of *ERG3*, which can be a biological response subsequent to overexpression, in which the inactivity of the enzyme encoded by *ERG3* derived from loss-of-function mutations results in a decrease in the synthesis of toxic sterol intermediates in the presence of azoles and, of course, favors the survival of the fungus [14,18,20]; However, studies do not focus their interest on the quantification of expression, so that the references that can provide valuable information on *ERG3* expression and resistance to azoles/fluconazole in *Candida* spp and especially in *C. glabrata* are scarce and the relationship between *ERG3* expression levels and azole resistance is not clear.

Exposing resistant strains to the antifungal decreased *ERG11* gene expression. The relative expression of the *ERG11* gene was low in both resistant and SDD strains, as well as low in strains exposed to the antifungal as in those not exposed; only three strains showed expression levels higher than 1, these results in the expression of the *ERG11* gene may be related as one of the biological conditions that explain the lower intrinsic susceptibility of *C. glabrata* to fluconazole, concerning other *Candida* species; since it is coherent to think that the low expression of the gene can affect the synthesis of lanosterol 14 alpha-demethylase and result in decreased pharmacological efficacy due to reduction of the pharmacological target [42].

There is little scientific evidence and the studies found do not report the specific values of *ERG11* expression in *C. glabrata*, in order to take the results as a reference and come closer to a better understanding of the phenomenon, which makes it necessary to conduct further research to broaden the conceptual reference.

The analyses in this study were conducted using colonisation strains taken from patients who had not undergone pharmacological interventions with fluconazole, and no information related to clinical variables was collected. These limitations should be considered in future research. In addition, it would be of particular interest to sequence the genetic material in order to not only identify the level of expression, but also to determine the type of genetic variations that may confer resistance.

5. Conclusions

The phenomenon of resistance is complex and depends on many factors specific to the fungus, the host, and the pharmacological intervention. It was possible to identify that strains resistant to fluconazole had an important expression of genes coding for drug efflux pumps, belonging to the ATP-Binding Cassette superfamily and that there was a much higher expression of the *ERG3* gene compared to the other genes involved in the analyses. This may be a particular characteristic of the *C. glabrata* species because of a comparatively low expression of the *ERG11* gene; these findings should motivate the continuity of research that favors the integral understanding of the adaptation-resistance dynamics to antimicrobials. To date, no similar work has attempted to understand the phenomenon of resistance through the integration of categorical and numerical variables derived from molecular observation and response to fluconazole in *C. glabrata*. Therefore, this contribution may be important for the scientific community so that it can be taken as a reference to involve complementary routes that integrate variables around predictive models that complement and streamline the analysis. Additionally, this type of molecular analysis allows integrating the explanatory scenario of the relationship between widely used antimicrobials and the biological activity represented by *C. glabrata* and considering that this type of study can help the scientific and clinical community to better understand the biological basis of resistance, to provide key elements in the resolution of the problem with a predictive, applicable, and therapeutic precision basis.

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